Serial No. 09/914,279

REMARKS:

Status of the Claims

1-6, 8-12, 14-50 and 55-70 are pending herein.

Claims 1-6, 8-12, 14-26, 55-68 and 70 are rejected.

According to the Office Action, claims 34-49 and 69 are withdrawn from consideration. Claims 27-33 and 50 are designated neither as rejected nor as withdrawn. However, these claims were previously withdrawn in response to Applicant's response to a restriction requirement dated August 26, 2003. Thus they are indicated herein as being withdrawn.

Claim 34 has been amended to introduce the non-ionic detergent limitation of claim 1. Support for this claim amendment can be found, for example, in original claim 7 and throughout the specification.

Claim Objections

Claim 62 is objected to as allegedly failing to depend upon another claim only in the alternative. This rejection is respectfully traversed.

Claim 62 presently reads as follows (emphasis added): "The microemulsion of ***any of*** claims 55-61..." It is believed that such language is proper alternative language. See, e.g., MPEP 608.01(n) I. A. Acceptable Multiple Dependent Claim Wording. Among the examples given as acceptable is the following: "Claim 10. A gadget as in ***any of*** claims 1-3 or 7-9, in which ---," which example contains language analogous to that used in presently pending claim 62.

Reconsideration and withdrawal of the objection to claim 62 are therefore requested.

Should the Examiner continue to object to claim 62, a suggestion regarding claim language that might be satisfactory would be appreciated.

Claim rejection under 35 USC § 112, first paragraph

Examined claims 1-6, 8-12, 14-26, 55-68 and 70 are rejected under 35 USC § 112, first paragraph, as allegedly not being enabling for microparticles having diameters outside the 10 nm

to 150 μm range given in the definition of the term "microparticle" given at page 12 of the specification. This rejection is respectfully traversed.

Moreover, as a result of the most recent restriction requirement, those claims that set forth microparticles have been withdrawn from consideration. Consequently, microparticles are not set forth in any of the rejected claims, i.e., claims 1-6, 8-12, 14-26, 55-68 and 70.

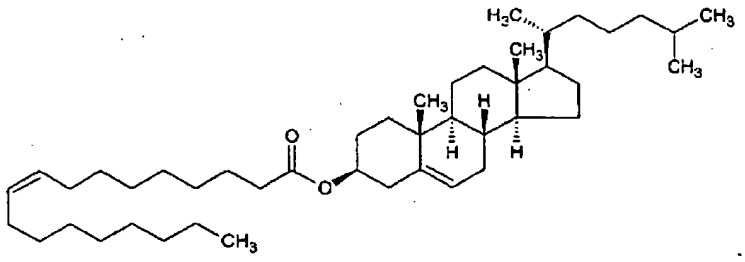
Reconsideration and withdrawal of the rejection of claims 1-6, 8-12, 14-26, 55-68 and 70 under 35 USC § 112, first paragraph, as allegedly being non-enabling for microparticles having diameters outside the range defined by Applicant, are therefore requested.

Claim rejection under 35 USC § 102

Claims 1-3, 9, 10, 19-21, 62, 64 and 67 are rejected under 35 USC § 102 as being anticipated by Hara et al., *Proc. Natl. Acad. Sci. USA*, Vol. 94, pp. 14547-14552 (Hara). This rejection is traversed.

For example, according to the procedure of Hara, negatively charged DNA was first complexed with a cationic lipid containing a quaternary amine head group (i.e., TC-Chol, 3-b-[*N*-(*N*9,*N*9,*N*9-trimethylethane)carbamoyl]-cholesterol). See Abstract and page 14548. The resulting hydrophobic complex was extracted by chloroform and then incorporated into reconstituted chylomicron remnant particles (RCR), which are composed of olive oil (a lipid), L-α-phosphatidyl choline (a phosopholipid), L-α-lysophosphatidyl choline (a phosopholipid), and cholesteryl oleate (a lipid). *Id.*

Each of the above is either charged or a lipid. None of the above is a non-ionic detergent as required by claim 1. The Office states that the limitation of a non-ionic detergent is met by cholesteryl oleate. However, cholesteryl oleate is actually a lipid. This is obvious from the structure of cholesteryl oleate (downloaded from chemexper.com):



. For further evidence, see merely, for instance, D.L.H. Rail et al., "Differential Contributions of Major Lipid Components of

Atheroma to Outcome of Cerebral Atheroembolism," *Stroke*, Vol. 12, No. 4, July-August 1981, 445-453 (attached) at page 445 ("[T]he main lipid constituents [of advanced atheromatous plaques] have been determined. Cholesterol esters (principally of oleic and linoleic acids) and cholesterol itself predominate...") and page 447 in which cholesterol oleate is listed under the heading of "Individual Lipids." See also J.M. Smaby et al., "Properties of cholesteryl oleate and triolein in mixed monolayers at the air-water interface," *Journal of Lipid Research*, Volume 19, 1978, 325-331 (attached), for example, the Abstract and page 326 where cholesteryl oleate is listed under the heading of "Lipids." Many other articles referring to cholesteryl oleate (or its synonym cholesterol oleate) as a lipid can be found, for instance, by searching PubMed at nih.gov.

For at least the above reasons, claim 1, and claims 2-3, 9, 10, 19-21 depending therefrom, are patentable over Hara.

Moreover, claims 62, 64 and 67 are patentable over Hara for the reasons set forth above with respect to claim 1.

Claims 4-6, 8, 11-12, 14-18, 22-26, 55-61, 63, 65, 66, 68 and 70 have not been rejected in view of the prior art. However, claims 62, 64 and 67, which depend from claims 55-61 are rejected in view of the prior art. It is not seen how the dependent claims can be rejected as anticipated in by the prior art, when the base claims from which they depend are allowable in view of the prior art. Clarification is respectfully requested.

Reconsideration and withdrawal of the rejection of claims 1-3, 9, 10, 19-21, 62, 64 and 67 under 35 USC § 102 are therefore requested.

CONCLUSION

It is respectfully submitted that all claims are presently in condition for allowance. Should the Examiner be of the view that an interview would expedite consideration of the application, request is made that the Examiner telephone the Applicants' attorney at (703) 433-0510 in order that any outstanding issues be resolved.

If there are any fees due and owing in respect to this amendment, the Examiner is authorized to charge such fees to deposit account number 50-1047.

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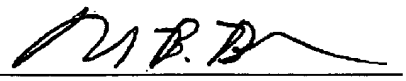

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Differential contributions of major lipid components of atheroma to outcome of cerebral atheroembolism. A study in an animal model

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Differential Contributions of Major Lipid Components of Atheroma to Outcome of Cerebral Atheroembolism

A Study in an Animal Model

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SUMMARY Cerebral atheroembolism, in which mainly lipid emboli are released from rupturing atheromatous plaques, may occur without apparent effect, or result in cerebral ischemia and infarction. The reasons behind these unpredictable consequences were sought in the interactions, *in vitro* and in an animal model, between the main lipid components of advanced plaques. Pure preparations of representative lipids were each harmless when embolized into the cerebral circulation. In contrast, combinations in proportions similar to those in advanced human plaques caused infarction, whether these were synthetic mixtures or extracts from plaques of the entire lipid fraction. The most important physical interaction between the lipids was aggregation of crystals by oils. Between cholesterol and the mainly liquid esters, this created *in vitro* a range of glutinous aggregates. Triglyceride lowered the melting point of esters, increasing their oiliness, and reduced the cohesiveness of aggregates in the face of operative mechanical forces through a fall in viscosity. Phospholipid, acting principally as an emulsifying agent, promoted dispersion of the oil, secondarily freeing the crystals from its aggregating effect. In the plaque, the balance of these factors will determine the size and number of particles likely to embolize, and, therefore, the clinical outcome should the plaque rupture.

Stroke, Vol 12, No 4, 1981

"CEREBRAL ATHEROEMBOLISM" is the term used for the release into the blood stream of mainly lipid emboli following rupture of advanced atheromatous plaques in vessels supplying the brain.[1] Recognition of its clinical importance[2, 3] hinges upon the argument that it is common. The precise prevalence is difficult to determine,[4] partly because it may occur without neurological sequelae[5] and remain unrecognized during life, and partly because difficulties in histological technique may prevent cerebral atheroemboli being found during postmortem examination.[6, 7] In one autopsy study of unselected patients,[8] atheromatous lesions were found in one or more of the major arteries to the brain in all patients over 50 years of age, and over two-thirds harbored advanced lesions. In patients with abdominal aortic atherosclerosis, distal atheroemboli were found postmortem in 12% of those with advanced disease.[9] Although minor variations in plaque constitution may be expected, there is no reason to suppose that the process of atheroembolism is subject to major regional differences once severe atheromatous disease is established locally.

Cerebral atheroembolism can be clinically silent,[5] but a number of published patient reports (e.g., Refs. 2, 4, 10, 11) demonstrate that cerebral ischemia, with or without infarction, may result. The nature of atheroemboli, and their behavior in the cerebral circulation, need to be better defined before their effects on the brain can be understood.

Chemical analysis of atheroemboli after release is seldom feasible, but the pultaceous contents of advanced atheromatous plaques from which they might arise have been described.[12, 13] By dry weight, up to 60–70% of this material is lipid.[14] Although to some extent varying from one plaque to another and from one region to another within the same plaque,[14] the main lipid constituents have been determined.[15, 16] Cholesterol esters (principally of oleic and linoleic acids) and cholesterol itself predominate (about 50% and 30% by weight respectively), with phospholipids (15%) and triglycerides (5%) present in smaller amounts. In early lesions (fatty streaks), cholesterol esters are laid down preferentially. Being in a liquid crystalline state (although fluid, maintaining a symmetrical molecular order[16] these are birefringent droplets. As the plaque develops, triglycerides accumulate and, together with a relative increase in amount of the more polyunsaturated cholesterol esters, reduce the melting point of the mixture[15, 16] which forms oily, isotropic droplets. Cholesterol is poorly soluble in this mixture, saturating the esters at a concentration of about 8%.[16] Progressive accumulation beyond this results in precipitation as cholesterol monohydrate crystals.[15, 17]

Phospholipid molecules have hydrophilic and hydrophobic groups.[18] In a mainly aqueous medium, such as is found in the center of a plaque, they form liposomes.[19] These structures are well characterized,[20] consisting of bilayers of phospholipid molecules oriented with hydrophilic poles at each surface, organized concentrically into multilamellar spheres; layers of water alternate with, and separate, the lipid layers. In the presence of cholesterol and its esters, liposomes have the capacity to incorporate, into the lipid bilayers, molecules of the former up to about 33% weight for weight of phospholipid.[18] The esters are very poorly taken up (2% weight for weight only), but, instead, may be more subject to the emulsifying effect of phospholipids, another property consequent upon the possession of hydrophilic and hydrophobic molecular poles.[18]

It has been suggested[21, 22] that the size and number of cholesterol crystals are the central determinants of

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the effects of atheroemboli because, acting as solid supporting skeletons and resisting lysis and removal, they maintain the integrity of emboli for long periods. We found that cholesterol crystals, dispersed into the cerebral circulation of rats or rabbits via the internal carotid artery, caused little hemodynamic disturbance, though aggregates of these crystals, if stable, were less innocuous.[9] Pure crystals, however, were usually disaggregated readily in the bloodstream, suggesting that physical interactions within the embolus between cholesterol and the other lipids, determining over-all physical state,[10] might be a more relevant factor than the crystals themselves to causation of infarction.

While it can be assumed that plaque contents do not alter their chemical composition with embolization, the same cannot be expected of their physical state after release into a turbulent, aqueous bloodstream. For this reason, theoretical extrapolation from plaque to embolus has limited scope and the effects in embolized lipid mixtures of these complex factors and relationships need to be studied directly. In the same way as with cholesterol,[9] therefore, the behavior of other important lipids has been observed in the cerebral vessels of the rat and rabbit. We have also looked at interactions between these lipids, both in these animal models and *in vitro*, with the primary aim of relating findings to their possible effects in man and, in particular, to their potential ability to cause cerebral infarction.

Methods

a) Animals

Young adult albino rats (350–450 g) were used in experimental procedures of 2 types (see below); New Zealand White rabbits were used in a few nonrecovery experiments. These methods have been fully described previously[1, 9] and outline details only are given here.

1) Open-skull Experiments

Rats were anesthetized with ethyl carbamate (Urethane, BDH). Blood pressure and body temperature were maintained. In each animal, a Portex cannula was introduced through a distal opening in the left external carotid artery so that its tip lay at the carotid bifurcation with the proximal stump of the external carotid totally occluded and flow from common to internal carotid unimpeded. Substances infused through such a cannula necessarily entered the internal carotid bloodstream. A dorsal craniotomy was carried out on the left side to expose an area approximately 10 × 5 mm of the dorsal cerebral surface which, after dural resection, was preserved under a pool of liquid paraffin at 37°C. The arteries from the middle cerebral trunk, with the largest about 80 μ in diameter, were viewed through a Zeiss Op-Mi 6 operating microscope at magnifications of up to 40 ×. The 3-way beam-splitting facility permitted simultaneous color video-monitoring, recorded on tape when appropriate, and still photography with a Nikon F2 camera body and motor drive to take frames singly or repetitively at up to 2.5 per second. Enhanced lighting and filters were available for fluorescein angiography, which demonstrated arterial, capillary and venous phases of perfusion and readily revealed areas of hemodynamic disturbance or extravasation.

In rabbits, a larger craniotomy opening could be made but all procedures were otherwise essentially similar.

In a few rats, the iris circulation was directly viewed through the microscope. The prominent vessels, supplied by the pterygopalatine branch of the internal carotid artery, were arterial arcades of much smaller caliber, about 20–30 μ.

Materials were embolized (see below) by infusion through the intracarotid cannula with either the cerebral or iris vessels under direct observation to control the quantity given. It was possible to monitor emboli arriving in the vessels and their progress distally, alterations in vessel caliber, changes in blood flow whether amounting to stasis or not, prolongation of circulation time, and areas of blood-brain barrier breakdown.

These experiments were terminal.

2) Recovery Experiments

These animals, anesthetized with pentobarbital sodium (Sagatal, May and Baker) intraperitoneally, were cannulated in the same way and then embolized immediately with predetermined quantities of material. The cannula was then withdrawn, the external carotid stump ligated, and the skin closed before recovery. Survivors were examined regularly for signs of neurological deficit and sacrificed between one and 7 days later. Brains were removed from all animals upon death (and, in some cases, parts of the lungs) and examined macroscopically and histologically.

b) Histology

Material for histological examination was fixed in 10% formol saline and then sectioned frozen (at 15 μ) or after paraffin embedding (5 to 15 μ). Frozen sections were examined microscopically either directly or through partially or fully crossed polarizing filters, or after staining for lipid with Sudan IV. Paraffin sections were stained with hematoxylin and eosin, Martius scarlet blue (for fibrin) or Luxol fast blue (for myelin).

c) Embolic Materials

Material of 2 types was used: mixed lipids extracted from human atheromatous plaques, and pure preparations of individual lipids representative of those most important in the plaque, singly or in combinations.

1) Lipid Extract

Suitable human aortae were obtained from the postmortem room. After removal of all coagulated debris from the intimal surface, pultaceous material was

scooped from the centers of advanced plaques. The lipid component was extracted from weighed quantities into chloroform and methanol, using the method described by Folch, Lees and Sloane Stanley,[23] and stored at −20°C under nitrogen until required.

2) Individual Lipids

Cholesterol esters (cholesterol oleate and linoleate), cholesterol (monohydrate crystals), phospholipid (phosphatidylcholine) and triglyceride (triolein) were obtained in a high degree of purity (usually 99%, Sigma London Chemical Co. or Lipid Products). Principal impurities in these preparations, other sterols and their esters of fatty acids of varying degrees of unsaturation, were unlikely to be of any significance in the proportions present. The lipids were dissolved in known concentration in chloroform and stored under nitrogen at −20°C until needed.

d) Preparation of Emboli

Measured quantities (usually 10 mg) of the required lipid materials (extract or one or more of the pure preparations) were added to 2 ml of saline-for-injection and separated from the chloroform or chloroform-methanol mixture in a rotary-evaporator at 37°C. When complete, this process left the lipids as particles suspended in saline, which were maintained at 37°C until embolized.

A variety of mixtures of the pure lipids were prepared either to be embolized or for assessment of the physical nature of the precipitated particles. These are summarized in the table. The particulate matter in the extract, as well as in these lipid mixtures, was examined by naked eye and then microscopically, both directly and through polarizing filters, and photographed. Agitation of the coverslip established how readily particles could be disaggregated. The appearance of individual particles was noted. Particle sizes were measured by micrometer and approximate numbers determined in a modified Nebueur counting chamber.

TABLE *Lipid Mixtures (Identified in Text by Nos. 1–18) Prepared for Embolization and/or Examination of Physical State*

		CE	Ch	TG	PL
1	E	—	—	—	100
2	E	—	20	—	80
3	E	—	50	—	50
4	E	—	50+ free crystals*	—	50
5	E	96	—	4	—
6	E	90	—	10	—
7	E	80	—	10	10
8	E	90	10	—	—
9		75	25	—	—
10	E	50	50	—	—
11		40	60	—	—
12	E	10	90	—	—
13		60	35	5	—
14		60	30	10	—
15		55	33	—	12
16	E	50	30	5	15
17		45	25	15	15
18	E	70	10	5	15

E in column 2 indicates preparations that were embolized. CE: cholesterol esters (equal proportions of oleate and linoleate); Ch: cholesterol; TG: triolein; PL: phosphatidylcholine. Figures in these columns indicate percentage of each component by weight. *In preparation 4, the proportion of cholesterol was increased by adding dispersed crystals in suspension ($5–6 \times 10^6$ in 2 ml of saline) after rotary evaporation of the cholesterol/phospholipid mixture. (See Steiner et al.[9] for details of preparation of crystals.)

Results

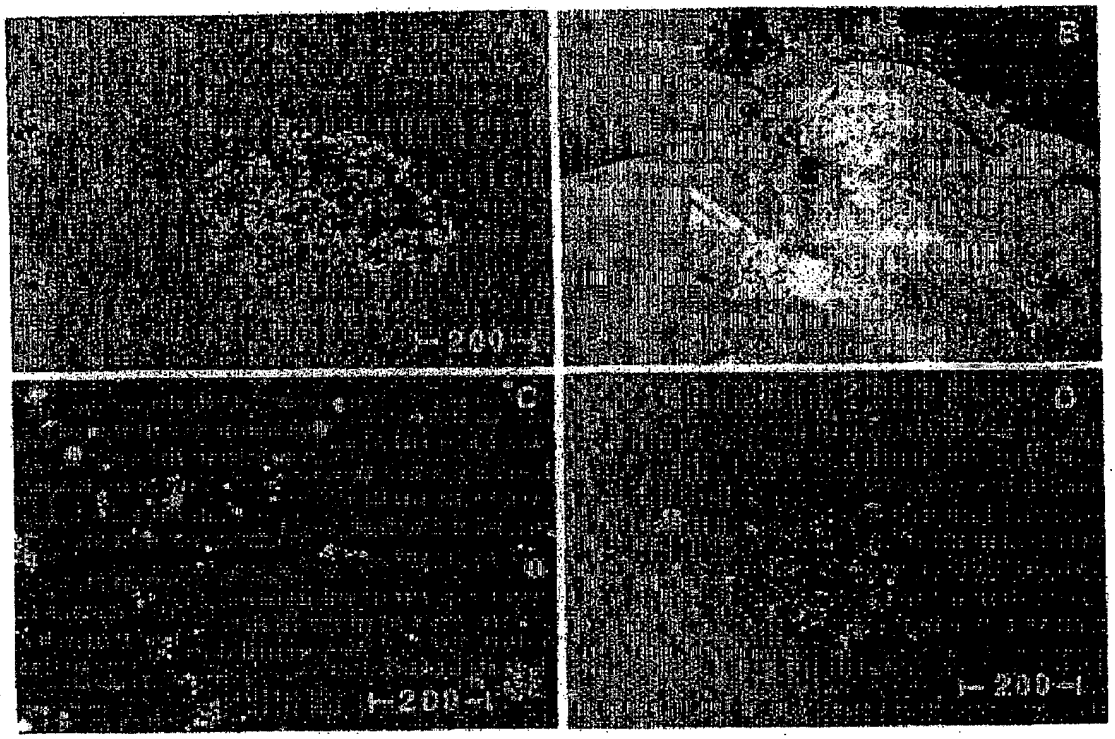
The lipid extract had the naked eye appearance of a greasy, watery liquid in which tiny suspended particles could be seen. It consisted of a suspension in saline of non-coalescent isotropic droplets, ranging downwards in size from 5 μ to less than 1 μ in diameter, and crystal aggregates in a concentration of the order of 10^6/ml; the latter, variable in both size and shape, ranged up to 400 μ greatest dimension (fig. 1*A*), but only 1 or 2% were greater than 100 μ and 90% were smaller than 30 μ. Perturbation of the coverslip, which partially broke up the aggregates, yielded single crystals up to 100 μ in length, though most were under 20 μ, together with further discrete droplets of oil and amorphous lipid material.

In the open-skull animals, the extract passed through the larger epicerebral arteries (50–80 μ diameter) and no disturbance of flow was apparent at this level. In the 30–40 μ side branches, some emboli lodged but discernment of flow disturbances in these vessels was hindered by their course into the cerebral substance. Fluorescein angiography (fig. 2) revealed no delay in the arterial phase of circulation but, subsequently, there was progressive extravasation of the albumen-bound dye deeply in the cortex (fig. 2*D*), from much smaller vessels. In the 20–30 μ vessels of the iris, although emboli were not seen, stasis was rapidly produced in some vessels and often occupied whole segments or evidence quadrants of the iris.

Animals recovering after infusion of 1–3 mg of this material showed no effect, or only transient limb paresis of less than 24 hours duration. Larger doses, up to 10 mg, produced contralateral hemiparesis in a high percentage of animals and postmortem examination revealed discrete ipsilateral infarcts involving cortex and white matter, often extending deeply as far as the ventricle (fig. 3). Sudan IV-stained frozen sections demonstrated lipid material in vessels of 20–30 μ diameter; occasional intravascular crystals were also seen through polarizing filters, but in neither case was there evidence of associated cellular reaction in the vessel wall to the emboli, or evidence of intraluminal thrombosis.

The appearance and behavior of the pure lipids and mixtures were dependent upon which lipids were included and on their relative proportions.

Phospholipid (phosphatidylcholine) suspended in



saline formed into liposomes in general smaller than 1 μ in overall diameter. About 2×10^6/ml were counted in the pure suspension (preparation 1 in the table). In preparations 2 to 4, increasing numbers of free crystals were apparent microscopically, with little tendency to aggregate and forming particles in the size range 5–100 μ greatest dimension. Embolization experiments with these mixtures were uneventful. Liposomes alone passed rapidly through the circulation without hindrance. Cholesterol crystal behavior was unaltered by the presence of liposomes and any aggregates reaching the epicerebral vessels could be seen breaking up readily if they lodged, with the individual crystals passing out of sight distally.

Cholesterol esters (1:1 mixture of oleate and linoleate) at 37°C were in a mixed state of crystals and liquid crystals. In the presence of triolein at a concentration of 4% (preparation 5), the microscopic appearances were of isotropic droplets, in size ranging from less than 1 μ to about 5 μ and in number about 0.5×10^6/ml, and some birefringent aggregates up to 30 μ in size. Increasing the proportion of triolein reduced the number of crystals further so that little birefringence was seen in preparation 6; droplets more readily coalesced and a few were up to 10 μ in size. Adding phospholipid (preparation 7) inhibited coalescence of droplets which were consequently smaller (less than .5 μ) and more numerous (about 1.5×10^6/ml).

These mixtures had little effect in acute animal preparations, with only brief stasis seen in smaller vessels such as those of the iris, sometimes in association with observed passage of small crystals. Of those animals injected with large quantities (10 mg) before withdrawal of anesthesia, most died within 6–12 hours, but material staining positively for lipid, and hemorrhagic interstitial and alveolar exudate, were prominent in the lungs. Those that did survive such treatment, and those injected with smaller quantities, recovered without neurological deficit; their brains showed no neuronal changes, though intra-arterial lipid in small amounts and occasional crystals could be demonstrated in vessels of the order of 20 μ in diameter.

FIGURE 1. *Comparative microscopic appearances of lipid extract and some of the synthetic lipid preparations listed in the table, photographed in excess of physiological saline, for technical convenience at 21°C. All scales (except in B) in microns.* A: *Total lipid extract from human aortic atheromatous material viewed through partially crossed polarizing filters. The large aggregate consists of birefringent crystals, mostly smaller than 20 μ in length, among other lipid material, partly amorphous, partly in droplets. Fewer than 2% of aggregates approached these dimensions and particles of more usual size are also seen. Many of the suspended non-coalescent isotropic droplets cannot be distinguished at this magnification.* B: *Mixture of cholesterol and cholesterol esters in approximately equal proportions (preparation 10: see table), with the appearance and consistency of a thick gum. The lipid/water interfaces are sharply demarcated with no admixture. Partially crossed polarising filters reveal a high degree of birefringence.* C: *Cholesterol esters and cholesterol in a ratio of 5:3, with phosphatidylcholine in a concentration of 12% (preparation 15: see table). The ester/cholesterol glutinous aggregate has been dispersed into microscopic particles which, however, retain their birefringence, starkly demonstrated by polarizing filters crossed almost fully.* D: *The mixture of esters and cholesterol, in a 5:3 ratio, with 5% of triglyceride and 15% of phospholipid (preparation 16: see table) approximates to the composition of the lipid extract, and the appearances are broadly similar (cf.* A). *Triolein impairs the phospholipid disaggregating effect demonstrated in* C *by conversion mainly of cholesterol esters to oil, so that birefringence is less prominent and there are oily droplets. As in* A, *the central aggregate, selected for demonstration, is at the upper extreme of the size spectrum; the smaller surrounding clusters are more typical, but many others are below the limits of resolution at this magnification.* E: *with triolein in greater excess than in* D *(preparation 17: see table), aggregates, though of similar size spectrum, appear less compact because of the more prominent and less viscous oily component. Removal of the polarizing filters allows the quantities of free isotropic droplets to be seen.*

After this preparatory examination of the major lipid components, cholesterol and cholesterol esters, the 2 were mixed, revealing a practical difficulty. As the proportion of cholesterol was increased (preparations 8 to 12), transition from the crystal/liquid crystal state of the esters to the solid crystalline form of cholesterol itself occurred through a series of sticky gum-like materials of increasing viscosity, many of which (particularly 9, 10 and 11) could not be handled because they stuck avidly to their containing flask. Microscopically, they were birefringent hydrophobic aggregates (fig. 1*B*) with little tendency to disperse unless one or other component was very greatly in excess to dictate behavior (preparations 8 and 12).

Preparations 9 to 11 could not usefully be embolized. Repeated attempts with the 1:1 mixture of esters and cholesterol (preparation 10) were unsuccessful because little of the material escaped the walls of the delivery apparatus. Mixtures in a 9:1 ratio (preparations 8 and 12), bearing little relationship to the proportions found in atheromatous material, were embolized only in a few recovery animals. Neither mixture was associated with neurological deficit or histological change.

Practical problems with ester/cholesterol mixtures were eased with the addition of triolein or of phosphatidylcholine. Increasing quantities of the former (preparations 13 and 14) produced greater numbers of isotropic droplets at the expense of the birefringent particle clumps. Particularly in 14, aggregates appeared more fluid, maintaining their integrity less well, so that mechanical agitation achieved more effective dispersal through the aqueous suspension. Addition of phospholipid to esters and cholesterol, on the other hand (preparation 15), produced ready and rapid dispersal of the birefringent aggregates into a suspension of smaller particles in much larger numbers (fig. 1*C*); many were smaller than 1 μ and, although the largest ranged up to 150 μ, 90% were under 10 μ greatest dimension and only 1–2% over 40 μ.

In mixtures of all 4 types of lipid, esters, cholesterol, phospholipid and triglyceride (preparations 16 to 18), the major interactions were to some extent predictable from the demonstrated properties of the simpler mixtures and the following account is a generalization. In the presence of triolein, phospholipid disaggregated the particles less effectively, which were somewhat larger in preparations 16 and 17 than in 15 (5% over 40 μ and 10% over 20 μ, though with similar upper and lower limits). Although particles appeared microscopically as crystal aggregates, birefringence was less prominent than in preparation 15; as in 13 and 14, which also included triolein, there were many isotropic droplets both within the aggregates and free. Aggregates were more compact in preparation 16 (fig. 1*D*) than in 17, in which they appeared loosely bound (fig. 1*E*). Preparation 18 consisted mainly of isotropic droplets though they might contain tiny crystals and there were some small crystal aggregates. Particles were well dispersed in numbers of about 2×10^6/ml and, though they were up to 50 μ in size, 90% were smaller than 10 μ greatest dimension.

With these mixtures, there was approximation to the lipid compositions of atheromatous gruel and plaque extract. Preparations 16 and 18 were embolized. While preparation 18, like 8 with a similarly low proportion of cholesterol, was without demonstrable effect in the animal model, preparation 16 was readily associated with cerebral infarction. Dosages as low as 2–3 mg (cf. lipid extract) produced infarcts that were small (0.25–1 mm) but often multiple (fig 4),

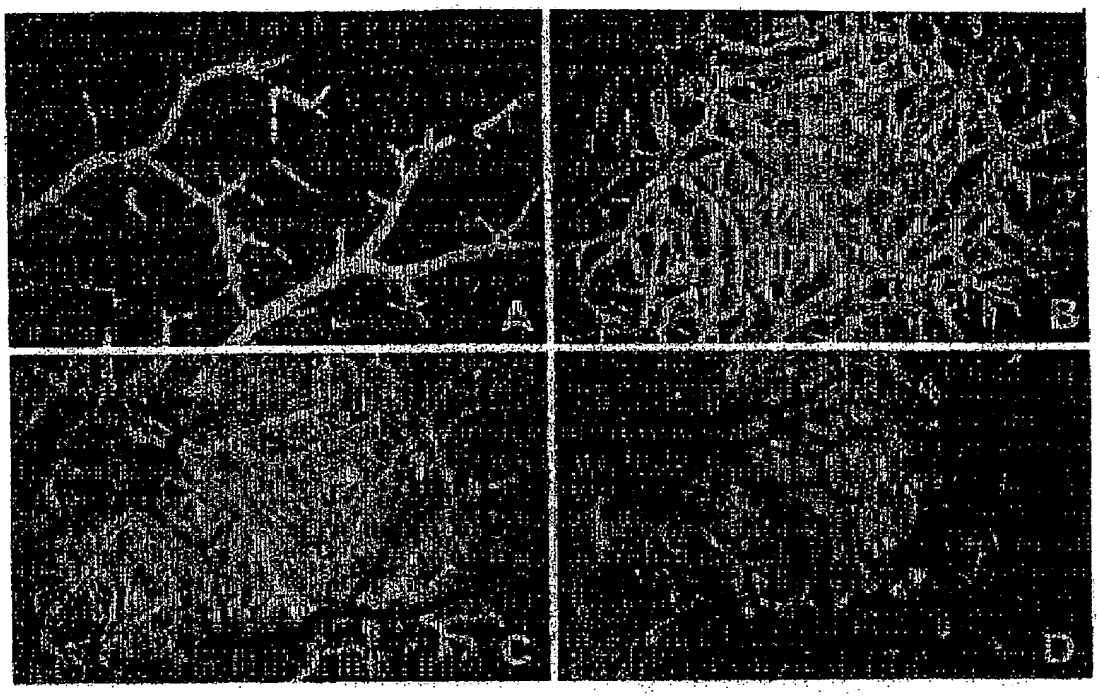


FIGURE 2. *Fluorescein angiogram of cerebral surface vessels viewed through left craniotomy in a rabbit. About 5 mg of lipid extract has been embolized by slow infusion into the ipsilateral internal carotid artery, but none has lodged in view.* A: *early arterial phase;* B: *capillary and early venous phase: no areas of ischemia or delayed perfusion are demonstrated;* C: *late venous phase: drainage is uniform, but small foci of perivascular fluorescence are appearing as the background fades;* D: *after clearance of the venous phase, extravasated fluorescein is brightly visible; its origins, widely dispersed within the fluorescein-perfused territory, are mainly deep in the cortex but those in clear focus are related to distal arterioles.*

FIGURE 3. *Coronal section of rat brain (left side) near mid-collicular level, stained with hematoxylin and eosin. Scale in mm. 10 mg of lipid extract infused into the ipsilateral carotid artery produced a discrete hemisphere infarct extending in each direction for several millimeters at the dorsal surface and deeply to the ventricular margin.*

occasionally bilateral, involving deep white matter and thalamic nuclei. Associated intravascular crystals were occasionally seen but intraluminal thrombosis was never in evidence.

Discussion

Our findings demonstrate that each of the main lipid components of atheromatous gruel, cholesterol, cholesterol esters and phospholipids, is harmless when dispersed in relatively pure form into the cerebral circulation via the internal carotid artery. They form innocuous emboli: either droplets, or particles, sufficiently small to pass through the capillaries without significant hindrance, or crystals of such shape that, though sometimes large enough to lodge at arteriolar or even arterial level, they do not create hemodynamic disturbance.[8] There is no vessel wall reaction and no thrombogenic effect. Nonetheless, both the total lipid extracted from gruel plaques, and synthetic mixtures prepared to simulate its constitution, caused infarction when embolized, and discussion needs to

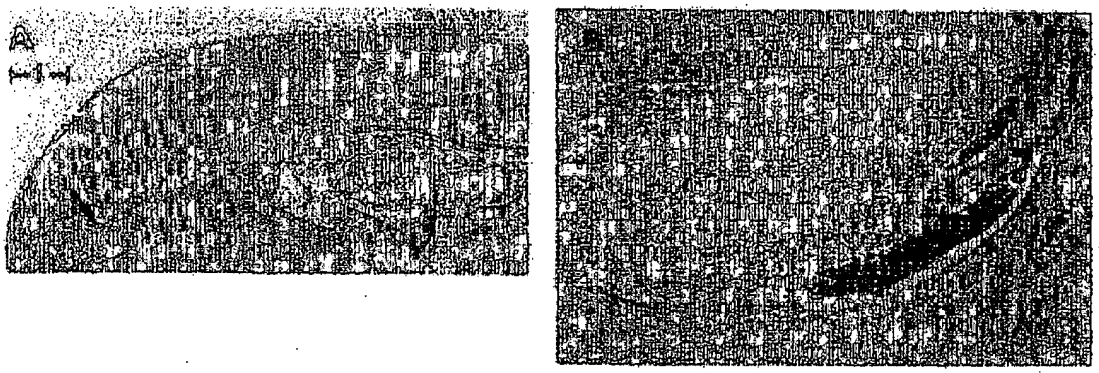


FIGURE 4. A: *Coronal section (dorsal part, left side) of rat brain at the level of the hippocampus and medial thalamic nuclei.*[34] *Hematoxylin and eosin stain. Scale 1 mm. Approximately 3 mg of a synthetic mixture of lipids (cholesterol, 30%; cholesterol oleate and linoleate, 25% each; phosphatidylcholine, 15%; triolein, 5% (preparation 16: table)), infused as in figure 3, produced numerous small infarcts throughout much of the cerebrum, some contralateral. This section shows hemisphere infarcts, particularly one dorsally near the mid line, one (arrowed) lateral to the nucleus caudatus putamen, and one dorsolateral to the lateral ventricle which involves the radiation of the corpus callosum; in addition, there is a relatively large hippocampal infarct, and other, smaller infarcts around the medial part of the medial thalamic nucleus have resulted in degeneration of the section.* B: *The lateral hemisphere infarct arrowed in* A *has been further enlarged about 10x.*

center around what property is possessed by these but not by any of their individual components that confers upon them their noxious quality.

A large proportion of the cholesterol is in crystalline form in the advanced plaque. When pure, such crystals have little tendency to aggregate in the cerebral circulation and are readily dispersed if they do.[9] In the presence of the other lipids, their behavior is demonstrably different and, undoubtedly, the key to this question is held in this interaction.

Much of this other lipid component exists in the plaque as oils.[10] These, by definition, are liquids immiscible with water, and form discrete droplets suspended in the aqueous medium of the plaque gruel or of the blood. The size of the droplets depends on a balance between their tendency to coalesce, a function primarily of surface tension, and the mainly mechanical forces promoting dispersal, such as agitation resulting from turbulence in the blood stream. Size and number are therefore inversely related (see below).

Cholesterol crystals, being hydrophobic, attract around them a layer of the oil which would otherwise form free droplets. Coalescence of this oil results in aggregation of the crystals and formation of oil/crystal mixtures with viscosities dependent on their relative proportions (cf. preparations 8–12). The size of such aggregates, though obviously dependent on crystal size, is now ultimately determined by the same factors of surface tension in the oil in competition with the mechanical dispersing forces, viscosity playing a modifying role.

Cholesterol and cholesterol ester mixtures, in combinations in which neither is in substantial excess (preparations 9–11), in practice form thick, gum-like substances so sticky that very few particles in suspension could be created. Mixtures of 1 : 1 cholesterol oleate and cholesterol linoleate at 37°C are in the form of crystals and liquid crystals,[10] the latter, to the extent of their liquid properties, behaving as an oil. Addition of increasing amounts of triglyceride, itself a low-viscosity oil, progressively lowers the melting point of the mixture into the ambient temperature range,[10] with consequent liquefaction and loss of birefringence. A similar effect on cholesterol itself[10] is much less important in view of its higher initial melting point (over 100°C in, for instance, a 3:5 mixture with cholesterol oleate). Because of overall reduction in viscosity, aggregates are more fluid in the presence of triglyceride, and relatively weakly bonded.

Phospholipids play a quite different role, equally important, in modifying particle size and aggregability. This may be most readily understood if the phospholipid molecule is regarded simply as an emulsifying agent, for this appears to be its major effect. Such agents, by reducing the surface tension of oil droplets in water, aid and maintain their dispersal (cf. preparation 7). The interaction of this effect with those of the other 3 components is depicted in figure 5, the whole concept being represented by a regular 4-faced pyramid, or tetrahedron. Removal of free cholesterol by incorporation within the liposome,[10] thereby reducing the crystalline component, is probably not without additional effect but of less importance in view of the quantities involved.

What is the relevance of these factors to the out-

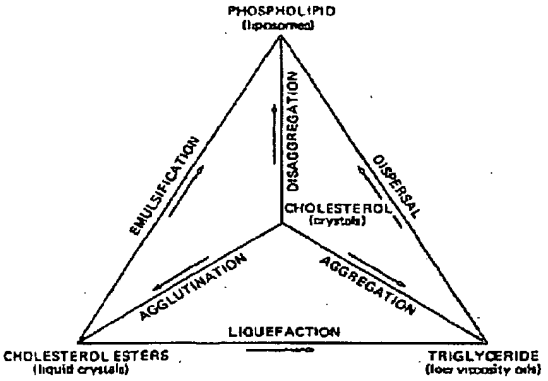


FIGURE 5. *Interactions between cholesterol, cholesterol esters, triglyceride and phospholipid, summarized as a tetrahedral concept. With the 4 lipids each represented at one of the 4 vertices, the principal effect of each on the others can be depicted theoretically on the 6 axes. In practice this requires some over-simplification. The interactions specified are those most relevant within the context of an atheroembolus; i.e., each indication of the effect of one lipid upon another assumes the modifying presence of the other two and, if considered in isolation, which is not the intention, may approximate variably to reality.*

come of atheroembolism? Apart from any harmful property inherent in the material of the embolus itself, the most important determinants of microembolic cerebral infarction are the size and number of the embolic particles.[18, 20] Size establishes the level, if any, of the arterial tree at which a sufficient proportion of the lumen is occupied by the embolus to compromise flow. Number, though of little importance until the critical minimum size is reached, then determines how many arterial channels are obstructed. Tissue ischemia will result only if enough channels of sufficient size are sufficiently compromised.

Phospholipids tend to reduce the size of ester/cholesterol/triglyceride particles, at the expense of increasing their number. Ultimately, this may result in large numbers of very small oil droplets, too small if embolized to embarrass the circulation even at capillary level (cf. preparation 18); total emulsification of the oil releases the cholesterol crystals in a non-aggregated state — demonstrated also to be harmless.[3] At an intermediate stage, the situation may be reached where a sufficient number of sufficiently large aggregates is the result. Preparation 16 approaches this state while at the same time approximating to the constitution of atheromatous gruel: this preparation, the total lipid extracted from atheromatous gruel, and atheromatous material itself,[21] all produced cerebral infarction when embolized. Here, the ambivalent effect of triglyceride becomes apparent. By increasing the oily component it expands the load to be emulsified. The excess oil induces coalescence of residual crystalline particles of cholesterol or its esters, and larger aggregates result than would otherwise form. The obvious disparity between the size of such particles embolized and the level of vessel obstruction is explained by poor cohesiveness due to the viscosity-lowering effect of triglyceride. Disaggregation in the bloodstream and molding of particles in the smaller vessels are both expected consequences of increased fluidity.

The influence of phospholipid on lipid emboli, therefore, may be largely protective against vessel obstruction and, to some extent, countered by that of triglyceride. However, in the absence of mechanical energy, emulsification depends on random molecular movement and is likely to be incomplete. Such conditions almost certainly obtain in the advanced atheromatous plaque, and may have important consequences. Whatever the overall composition, differing degrees of emulsification throughout the plaque will unpredictably affect the size, number and nature of particles. Since the total number released by a single rupturing plaque may be many millions, such could be the effect on the target tissue if, say, just 1% reach a critical size that the nature of the remaining 99% is, for practical purposes, immaterial. This is a very important concept.

These various considerations indicate a cause of the apparently disparate sequelae of cerebral atheroembolism. Critically dependent upon possibly minor constitutional changes among the lipids, effects may well vary substantially from one affected individual to another. In routine clinical practice, the spectrum may be concealed. At one end, it seems certain, effects are insignificant and, if seen at postmortem examination, such cases may be those with ulcerated plaques in the neck vessels but no history or signs of ischemic brain damage. Towards the other extreme are the reported cases of atheroembolic cerebral infarction with obvious atheromatous material in the cerebral blood vessels at autopsy (e.g., Ref. 10). Intermediate in the spectrum is the unknown number of patients in whom symptoms of transient cerebral ischemia result but are not reported, or the possibility of an association is not appreciated. In addition, a large number of patients are seen postmortem with small cerebral infarcts in whom evidence suggesting the immediate cause is either inadequately sought[6, 9] or shown to be absent. Even macroemboli can disappear within hours of lodging;[17] it is therefore highly probable that microemboli initially associated with a non-fatal infarct will not be present after the delay of months or years that is likely before autopsy becomes possible.

Infarcts resulting from multiple microemboli contrast with infarction due to macroembolic vessel occlusion not only in pathogenesis[20, 30] and the nature of the embolic material. Occlusion of small, distal vessels, with the collateral circulation similarly affected, may cause more profound tissue ischemia than blockage of a large (proximal) artery,[20] whether by a macroembolus or atherosclerotic lesion, or otherwise. Such distal obstruction was characteristic of embolization with the total-lipid extract: in open-skull animals, extravasation of albumen-bound fluorescein from small vessels signified damage at that level

(figure 2*D*); in recovery experiments, evidence of lipid was seen histologically in 20–30 μ vessels.

Atheroemboli, having larger aggregated cholesterol crystals than those obtained by lipid extraction, are able to obstruct vessels of the order of 80–100 μ in diameter.[10] If, in keeping with our findings, they also block those of 20–30 μ they may be unique among the various spontaneously arising emboli in threatening both major terminal anastomotic beds, pial and precapillary.[10] Since atheroemboli often occur where blood flow is compromised already by the proximal atheromatous lesions from which they originate, their potential ability to curtail supply below the limits compatible with tissue viability is only too clear. The expected result is multiple small infarcts (fig. 4), patchily widespread in accordance with the vagaries of embolic distribution among collateral channels by laminar flow. The neurological consequences, for these reasons alone, would be unpredictably variable, governed not only by this chance distribution but also on the basis of availability of neuronal reserves that may be silently depleted.

Acknowledgment

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Properties of cholesteryl oleate and triolein in mixed monolayers at the air–water interface

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Abstract The properties of cholesteryl oleate and triolein in mixed monolayers at the air–water interface have been measured between 24 and 37°C. Analysis of force–area curves obtained as a function of the mol fraction of cholesteryl oleate indicates that at relatively low surface pressures these compounds are miscible in two dimensions up to a limit of about 0.5 mol fraction. At higher pressures either cholesteryl oleate or both lipids are expelled from the monolayer to form a bulk phase which is in rapid equilibrium with the surface phase. In the monolayer phase, orientation of the ester function of cholesteryl oleate is toward the aqueous phase, interaction with triolein is minimal, and packing is uniform over the solubility range. This, together with the susceptibility of the cholesteryl oleate to enzymatic hydrolysis, suggests the applicability of monolayer systems to the study of cholesterol esterase activity. Comparison of our results with the bulk properties of these lipids suggests that the expelled cholesteryl oleate exists as a smectic mesophase and thus the system may provide a model for studying the transfer of molecules between the interior and surface of lipid deposits of the type found in atherosclerotic lesions.

Supplementary key words miscibility · phase transition · liquid crystal · cholesterol esterase

Long chain cholesteryl esters are relatively nonpolar molecules (1) which exhibit limited solubility in binary mixtures with more polar lipids such as triglycerides (2, 3) and phospholipids (4). In ternary systems containing a low concentration of water, the solubility of the cholesteryl esters in the lamellar lipid phase is even lower, but it increases when the water content of the system is above 15% by weight. Concomitant studies with polarizing light microscopy, calorimetry, X-ray diffraction, and monolayer expansion suggest that, at the higher water concentration, the cholesteryl ester is located at the lipid–water interface (5). This conclusion is reinforced by enzymatic studies which show that small amounts of cholesteryl oleate incorporated into unilamellar lecithin liposomes are hydrolyzed by extracts from liver and aorta (6, 7).

At an air–water interface the behavior of cholesteryl esters depends largely on the structure of the acyl function. The short chain esters, cholesteryl formate (8) and acetate (9, 10), readily form monolayers which, at low surface pressures, exhibit force–area isotherms quite similar to that of cholesterol alone. This similarity has been taken as an indication that the acyl function protrudes into the aqueous phase and thus does not contribute to the area occupied by the molecule in the monolayer. Cholesteryl butyrate, which has a longer acyl chain, exhibits an unstable isotherm on an aqueous subphase and collapses at 7 dynes per cm (approximately 42 Å² per molecule). Making the subphase 2 M in sodium chloride increases the stability of the film while changing the limiting area only slightly (11). The similarity of these areas to that of cholesterol alone has led to the conclusion that at collapse the butyl group is forced down into the aqueous phase. Increasing the acyl chain to six carbons results in even more unstable films, even on 2 M sodium chloride.

The surface properties of long chain cholesteryl esters, which are more relevant to biological systems, have also been investigated. Both saturated and unsaturated esters yield pressure–area curves when compressed at an air–water interface, but the monolayers are unstable and give limiting areas for these molecules at collapse in the range of 25–30 Å² per molecule, values far below the collapse area of cholesterol alone (9, 10, 12). For the esters containing oleate, linoleate, linolenate, and arachidonate, it has been suggested that the isotherms are produced by oxidation products rather than by the esters themselves (9). In mixed monolayers with more surface active lipids such as lecithin, triglycerides, or cholesterol, long chain cholesteryl esters produce an expansion effect, indicating their presence in the monolayer phase (9, 10, 12). As the mixed monolayers are compressed, the cholesteryl ester is extruded into a bulk phase at a pressure that is dependent upon its solubility in the other lipids.

The relevance of the surface behavior of cholesteryl esters to an understanding of their deposition and

removal from the arterial wall has prompted us to study the expansion effects in more detail. Noting, as previous authors have (10), the behavioral similarities between these lipids and apolar hydrocarbons, we have applied to our data the mathematical treatment employed by Davis, Krahl, and Clowes (13) and Clowes (14), and later by Snart (15), for mixed monolayers containing hydrocarbons. Our results show a two-dimensional miscibility (16, 17) between cholesteryl oleate and triolein in the range from 24 to 37°C and provide information about the arrangement of molecules in both the surface and collapsed phases.

MATERIALS AND METHODS

Reagents

Lipids. Cholesteryl oleate and triolein were obtained from Nu-Chek Prep, Elysian, MN. They had a stated purity of 99+% and gave a single spot when analyzed by thin-layer chromatography with a solvent system of petroleum ether–ether–acetic acid 85:15:1 (v/v/v).

[^{3}H]Cholesteryl oleate was synthesized on a micro scale from oleoyl chloride (Nu-Chek Prep), cholesterol (Nu-Chek Prep), and [1,2-^{3}H]cholesterol (New England Nuclear; lot 853-154, 60 Ci/mmol) as previously described (18).

Spreading solvent. Petroleum ether (bp 60–70°C) was stirred with 98% sulfuric acid for 20 hr and washed once with water, once with 0.1 M sodium bicarbonate, and twice with water. The organic layer was dried overnight over calcium chloride and distilled from calcium hydride. The product had a boiling range of 65–68°C and gave no measurable force–area curve when spread at twice the level normally used for force–area measurements (see below).

Cholesterol esterase. The preparation of this enzyme from porcine pancreas has been previously described. The sample used was 94% pure as determined by photodensitometry of stained gels from polyacrylamide gel electrophoresis (18).

Other chemicals. All other compounds were reagent grade and were used without further purification.

Experimental procedures

Force–area curves. Surface pressure–area determinations were made using a Lauda recording film balance (Brinkmann Instruments, Westbury, NY). This instrument is a Langmuir type balance in which surface pressure is measured using a floating barrier attached to an inductive linear transducer. Unless otherwise indicated, lipids were spread in 50 μl of petroleum ether on to a 10 mM potassium phosphate, 0.1 M sodium chloride subphase, pH 6.6. After standing at 195–240 Å^2/molecule of triolein for 3 min, the monolayer was compressed at approximately 15 Å^2/min per molecule of triolein to an area/molecule of 90 Å^2, and then expanded to the original area at the same rate. Force–area curves were obtained by recording surface pressure vs. area/molecule of triolein during the compression–expansion cycle.

Surface tension measurements. During enzymatic digestion of mixed monolayers, surface tension was monitored at 24°C using a duNoüy ring attached to a Cahn RG recording electrobalance (19) equipped with a T-Y strip chart recorder. Maximum pull on the ring was maintained by manually moving the balance, mounted on a rack and pinion transport, up and down as required.

Enzymatic digestion of monolayers. Hydrolysis was measured in a circular Teflon trough with a diameter of 4 cm and a volume of 10 ml. For each assay 10 mM phosphate buffer, 0.1 M NaCl, pH 7.5, was added, the surface was cleaned by aspiration, and a solution of triolein and cholesteryl oleate in petroleum ether containing approximately 1.5×10^4 dpm of [^{3}H]cholesteryl oleate was added to give a surface pressure of approximately 4 dynes/cm. After allowing 3 min for complete evaporation of solvent, the solution was magnetically stirred, cholesterol esterase (85 μg in 100 μl of buffer) was added and after 1 min stirring was stopped. After 30 min the monolayer was collected and percent hydrolysis of the cholesteryl ester was determined by thin-layer chromatography–scintillation counting as previously described for the hydrolysis of glycerides by pancreatic lipase (19).

Measurement of radioactivity. Radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer and a toluene–Triton X-100-based scintillation fluid (20).

RESULTS

The ability of cholesteryl oleate to increase the apparent area/molecule of triolein in monolayers is shown in **Fig. 1a–c**. Such expansion is in qualitative agreement with previous studies (9, 10, 12) and shows that the cholesteryl ester is occupying space in the monolayer. As the surface pressure is increased in the region from approximately 0.15 to 0.5 mol fraction of cholesteryl oleate, each curve shows a phase transition above which the curves form a common

envelope. This is consistent with the miscibility of cholesteryl oleate and triolein in the surface phase where the phase transition pressure, or critical pressure, is the point at which cholesteryl oleate is ejected to form a bulk phase (16, 17). Although not shown in Fig. 1a–c, the envelope curve approached the abscissa at large molecular areas, showing that the equilibrium spreading pressure of cholesteryl oleate is near zero dynes/cm. The possible existence of a mixed, gaseous phase at large areas is not excluded, but our instrument is not sufficiently sensitive to study this region of the force–area curves.

If the triolein–cholesteryl oleate system behaves ideally, the envelope curve should extend up to and collapse at the same point as triolein. As the figures show, increasing the mol fraction of cholesteryl oleate decreases the collapse pressure toward a lower limit. Similar behavior has been observed for hydrocarbons in monolayers of cholesterol (14) and should indicate the formation at higher pressure of a collapsed phase

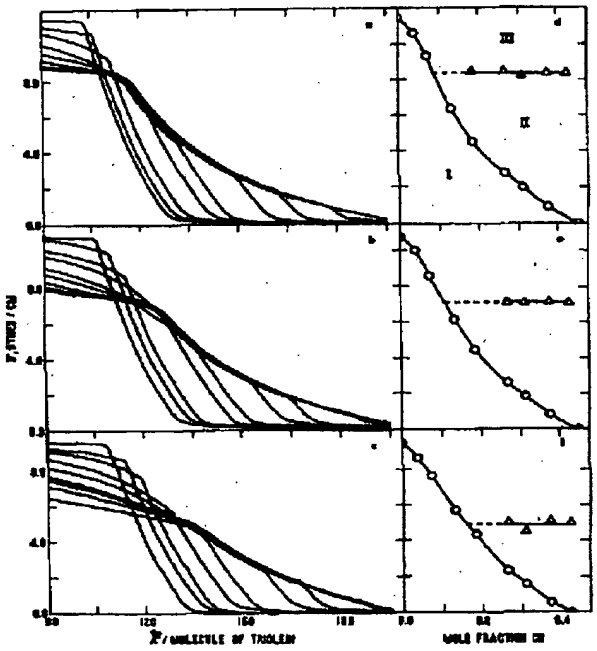

Fig. 1. Force–area curves (a–c) and monolayer phase diagrams (d–f) for cholesteryl oleate (CO) triolein mixtures. Subphase was 0.01 M potassium phosphate–0.10 M NaCl, pH 6.6. Temperatures were 24°C (a and d), 30°C (b and e) and 37°C (c and f). For each set of curves the mol fractions of cholesteryl oleate were, from left to right, 0, 0.037, 0.072, 0.133, 0.186, 0.265, 0.311, 0.374, 0.425, and the number of triolein molecules was held constant at 2.41×10^{16}. Circles (d–f) show critical pressures and triangles are collapse pressures for curves exhibiting two phase transitions. Data are from Fig. 1, a–c.

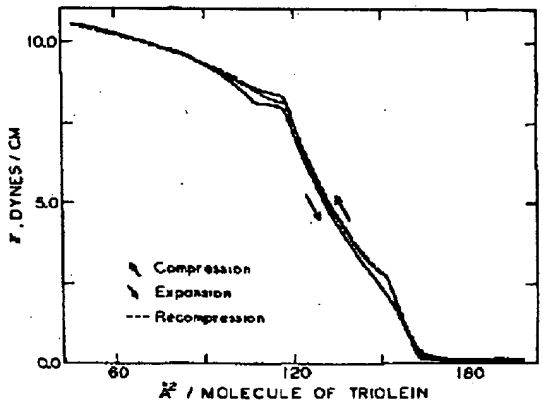

Fig. 2. Reversibility of collapse for a typical cholesteryl oleate–triolein monolayer. Subphase was 0.01 M potassium phosphate–0.10 M NaCl, pH 6.6, 24°C. The mol fraction of cholesteryl oleate was 0.267. Initial compression is upper solid line; expansion curve is lower solid line; and recompression is dashed line.

containing both triolein and cholesteryl oleate (17). A consequence of the bulk phase solubility of these lipids is that the force–area curves obtained at the lower mol fractions of cholesteryl oleate do not show a well defined critical pressure apart from the collapse pressure.

If the interpretation of the data shown in Fig. 1 is correct, the surface and bulk phases should be in equilibrium (16, 17) and the force–area curves should, therefore, be reversible. The curves in **Fig. 2** show that this criterion is fulfilled. A mixed monolayer was formed at an area of 210 Å²/molecule of triolein and then compressed to 45 Å²/molecule. The surface was then expanded to its original area and recompressed. There is a small hysteresis observable in comparing expansion with compression but recompression of the monolayer gives a force–area curve essentially identical to the initial compression curve. The near identity of two compression curves also shows that molecules were not lost during the experiment through barrier leakage or dissolution into the aqueous phase. Expansion curves are not shown for each compression curve shown in Fig. 1, but they were recorded. At 24 and 30°C, results similar to those given above were obtained, whereas, at 37°C, the expansion curves frequently gave values up to 0.5 dyne/cm below zero at large areas. This reflects an observed instability of the zero point at this temperature.

In addition to measurement of reversibility, other control experiments were performed to test the stability and reproducibility of the system. The following changes in procedure had no significant

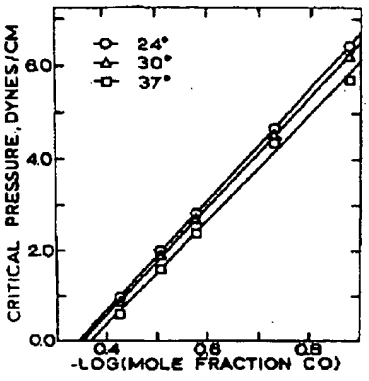

Fig. 3. Critical pressures vs. negative logarithm of the mol fraction of cholesteryl oleate at 24°C (circles), 30°C (triangles) and 37°C (squares). Lines were fitted by method of least squares. Data are taken from Fig. 1, a–c.

effects on either compression or expansion curves obtained at 24°C: *a*) varying the compression speed from 8.0 to 33.6 Å²/min per molecule of triolein; *b*) initially spreading the monolayer at 360 Å²/molecule instead of 195–240 Å²/molecule; *c*) initiating compression after 14 min instead of the usual 3 min; *d*) allowing the monolayer to stand at 90 Å²/molecule of triolein before recording the expansion curves; and *e*) spreading the monolayer at 45 Å²/molecule of triolein instead of 195–240 Å², expanding it to 195 Å²/molecule then compressing it, and reexpanding it. Compression–expansion curves determined at 24°C on several days with different solutions, each containing 0.186 mol fraction of cholesteryl oleate, were reproducible with percent standard deviations for area/molecule of triolein at a given surface pressure of 0.7% for compression and 0.9% for expansion, both below and above the critical pressure. The collapse pressures measured at 33 Å²/molecule of triolein had a percent standard deviation of 0.5%. The pH dependency of the system was obtained by measuring force–area curves for a mixture containing 0.261 mol fraction of cholesteryl oleate on subphases of 10 mM potassium phosphate, 0.1 M NaCl at pH values from 3.5 to 9.5 in 1.0 pH increments. The set of curves obtained (not shown) was essentially identical with percent standard deviations at 3.0 and 7.0 dynes/cm of 1.0 and 1.1 percent. All curves were reversible. Thus, the data from Figs. 1 and 2, together with the control experiments, show that at temperatures from 24–37°C and over a wide range of pH values, cholesteryl oleate and triolein form a miscible surface phase which, within the time course of our experiments, exists in equilibrium with a bulk phase of cholesteryl oleate, or cholesteryl oleate and triolein, depending on the surface pressure.

From the interpretation given above of the data from Figs. 1a–c, a phase diagram can be constructed for each temperature. These are shown in Fig. 1d–f and each shows three distinct regions. In region I, only a single, mixed surface phase is present; in region II, both the surface phase and a cholesteryl oleate bulk phase coexist; and in region III, the surface phase can coexist with a mixed triolein–cholesteryl oleate bulk phase.

For such a miscible system at equilibrium, it has been shown that a plot of the negative $\log_{10}$ of the mol fraction of the expressed component, cholesteryl oleate, vs. the critical pressure should be linear for values of the mol fraction over which the activity coefficient of the expressed component is constant (16, 17). Usually, this occurs near saturation. **Fig. 3** shows that, for mixed monolayers of triolein and cholesteryl oleate, this relationship is obeyed not only near saturation but at all values of mole fraction of cholesteryl oleate above 0.13. The data are from Fig. 1a–c and the coefficient of correlation for each line was ≥0.99. The slope of each line should be $2.303RT/A_e$ where R is the gas constant, T is the absolute temperature and A_e is the molecular area of cholesteryl oleate at its point of expression from the monolayer. In addition, the intercept of the line with the equilibrium spreading pressure of the expressed component gives the solubility limit for that component in the mixed monolayers (16, 17). The data show that pure cholesteryl ester in the monolayer phase does not exhibit a measurable spreading pressure; hence, the negative log of the solubility limit is given by the x-intercept of the line. **Table 1** shows the values of A_e and the solubility limits calculated from the slopes and intercepts of the lines shown in Fig. 3. The solubility decreases only slightly with temperature and, as would be expected, increasing temperature causes an expansion of the area occupied by cholesteryl oleate at collapse.

The free energy required to expel cholesteryl oleate from the mixed monolayer can be calculated for each

TABLE 1. Solubility limit and area/molecule of cholesteryl oleate at the critical pressure for triolein–cholesteryl oleate monolayers

Parameter, °C	Temperature, °C		
	24	30	37
Solubility limit, mol fraction	0.452	0.445	0.427
Å²/molecule of cholesteryl oleate	78.2	81.2	85.6

Data are calculated from the slopes and intercepts of the lines in Fig. 3 as described in the text.

TABLE 2. Work required to eject cholesteryl oleate from triolein–cholesteryl oleate monolayers

Mol Fraction Cholesteryl Oleate	Temperature 24°C π_c Dynes/cm	24°C ΔF Cal/mol	30°C π_c Dynes/cm	30°C ΔF Cal/mol	37°C π_c Dynes/cm	37°C ΔF Cal/mol
0.183	6.40	720	6.20	725	5.70	702
0.186	4.70	529	4.50	526	4.35	536
0.265	2.80	315	2.65	310	2.35	290
0.311	2.00	225	1.90	222	1.65	203
0.374	0.95	107	0.85	99	0.60	74

Data are taken from Fig. 1, a–c. ΔF was calculated as $NA_c'\pi_c$ where N is Avagadro's number, A_c' is the molecular area of cholesteryl oleate at the critical pressure, π_c. A_c' was calculated as the difference between the molecular areas of the mixed monolayer and triolein alone at π_c, divided by the mol fraction of cholesteryl oleate in the monolayer.

curve from the relationship, $\Delta F = NA_c'\pi_c$, where N is Avagadro's number and A_c' is the molecular area of cholesteryl ester at the critical pressure, π_c (14). **Table 2** shows that, as the mol fraction of cholesteryl oleate in the monolayer is increased, the free energy change decreases from approximately 0.7 to 0.1 Kcal/mol, indicating a relatively weak interaction between cholesteryl oleate and triolein in the monolayer.

Although the system behaves ideally from about 0.1 to 0.5 mol fraction of cholesteryl oleate the points from 0.0 to 0.1 mol fraction do not fit the lines shown in Fig. 3. The deviation is probably due to the solubility of the triolein in the bulk cholesteryl oleate phase, but could be due to the existence of a different molecular arrangement of triolein and cholesteryl oleate in the monolayer at low mol fraction of cholesteryl oleate. To test this hypothesis, we determined the average area/molecule of lipid in the monolayer for 0.5 dyne/cm intervals from 0.5 to 6.0 dynes/cm as a function of the mol fraction of cholesteryl oleate. It can be readily shown that if the packing of the molecules is uniform over the entire solubility range then at any surface pressure below the critical pressure a plot of average area/molecule of lipid vs. mol fraction will be linear, up to a 1:1 ratio of cholesteryl oleate to triolein. Each data set was reasonably linear (coefficient of correlation ≥0.97) and **Fig. 4** shows three such plots at 24°C for surface pressures of 1.5, 2.5, and 5 dynes/cm. This linearity indicates that at any surface pressure the packing of molecules in the monolayer is the same over the entire solubility range.

The apparent area per molecule of cholesteryl oleate in the monolayer can be obtained at any pressure by mathematical extrapolation of the line to 1.0 mol fraction. Using the apparent molecular areas determined every 0.5 dynes/cm, a force–area curve for cholesteryl oleate was calculated at each temperature as shown in **Fig. 5** (circles). Shown for comparison (solid line) for each is the predicted force–area curve for cholesteryl oleate which was calculated as the sum of measured area/molecule of cholesterol and one-third of the measured area/molecule of triolein at each pressure and temperature. In each case, the agreement between curves is good, considering the limited number of points, the extrapolation necessary to determine the actual force–area curve, and the implicit assumptions necessary to calculate the predicted curve. This agreement, together with the low free energies of solution, suggests that "condensation" between cholesteryl oleate and triolein is minimal and that the cholesterol and acyl moieties of cholesteryl oleate in the monolayer are oriented as they would be in more polar molecules, i.e., with the ester group facing the aqueous phase and the apolar groups away from it.

To test this orientation hypothesis, we measured the susceptibility of [^{3}H]cholesteryl oleate in the mixed monolayer to hydrolysis of cholesterol esterase from

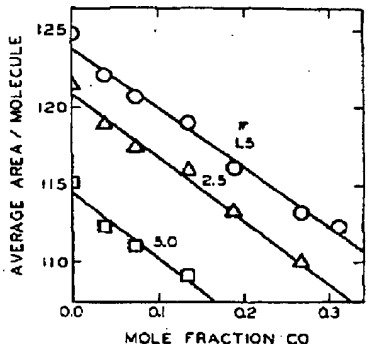

Fig. 4. Typical plots of average area/molecule for triolein–cholesteryl oleate (CO) mixtures at 24°C. Surface pressures (π) were 1.5, 2.5, and 5.0 dynes/cm. See Fig. 1 for experimental conditions and raw data.

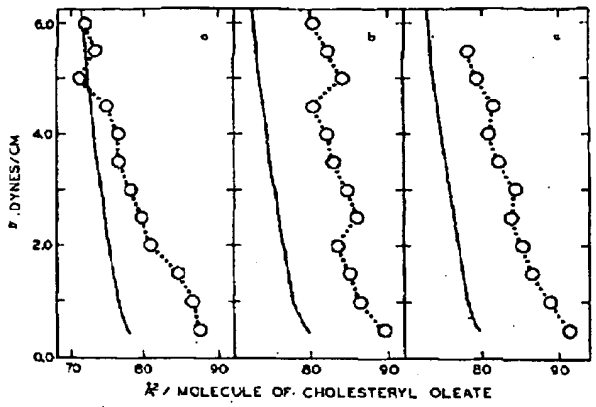

Fig. 5. Extrapolated and predicted force–area curves for cholesteryl oleate. Predicted curves (solid lines) were generated by summing areas/molecule of cholesterol and ½ triolein obtained under conditions identical to those of Fig. 1. Extrapolated curves were obtained from plots of the type shown in Fig. 4 as described in the text. Left to right, 24°C, 30°C, and 37°C.

porcine pancreas in the aqueous phase. This enzyme readily hydrolyzes trioctanoin and triolein in monolayers at an air–water interface[1] and should hydrolyze cholesteryl oleate in a mixed monolayer if the ester assumes an orientation at the interface similar to that of the triglyceride. The mol fraction of cholesteryl oleate was 0.186, a value that insured that the initial pressure of 4 dynes/cm was below the critical pressure for that mixture. After 30 min incubation with enzyme at 24°C, 86% of the cholesteryl ester was hydrolyzed, whereas less than 1% was consumed in the absence of enzyme. Essentially identical results (75% hydrolysis) were obtained with dioleyl ethane diol, which forms mixed monolayers with cholesteryl oleate under the same conditions but cannot be attacked by the enzyme. These data support the results of the physical studies and indicate that the ester function is oriented toward the air–water interface as it is for triglycerides.

DISCUSSION

Our results show clearly that cholesteryl oleate and triolein are miscible at the air–water interface. Not only do the combined curves form an envelope, but also the system is completely reversible and independent of the previous history of the monolayer.

The solubility of cholesteryl oleate in triolein approaches 1:1 at all temperatures studied. This value is considerably higher than was observed for the ternary system of lecithin–cholesteryl linoleate–water where one part in 32 of the cholesteryl ester could be dissolved in lecithin bilayers in the presence of excess water (5). This difference in solubility limits probably reflects the difference in packing densities between lecithin in a bilayer phase and triolein in the monolayer system. In the monolayers the highest solubilities are at surface pressures approaching zero dynes/cm whereas in the erythrocyte membrane, a natural bilayer, packing densities are comparable to those in a phospholipid monolayer at 31–34 dynes/cm (21).

As in the bilayer system, our physical and enzymatic data indicate that cholesteryl oleate in the monolayer phase has its ester function oriented toward the aqueous phase. Furthermore, the agreement between the predicted force–area curve for cholesteryl oleate and that from extrapolation indicates that the overall orientation of the molecules differs little from that of cholesterol and the acyl moieties of glycerides under equivalent conditions. Our values of 70–90 Å² per molecule for cholesteryl oleate do, however, differ markedly from the 25–30 Å² per molecule obtained with cholesteryl esters alone or in mixed monolayers containing cholesterol (12), indicating that the nature of the other lipid has a large influence on the state and/or orientation of the cholesteryl ester at the interface.

The linearity of the average area plots shows that the packing of the molecules is uniform over the entire miscibility range and the similar solubility limits and A_c values at 24, 30, and 37°C show that there is no major change in that arrangement with temperature. Likewise, alteration of subphase pH has, as would be expected for a neutral monolayer, a negligible effect. This uniformity of molecular arrangement over a range of experimental conditions together with the ability to vary monolayer composition and packing density independently suggest that monolayer systems of the type described will be valuable tools for studying the hydrolysis of cholesteryl esters by water soluble enzymes.

The reversibility of the force–area curves shows that the molecules in the collapsed phase are in rapid equilibrium with those in the surface phase. This suggests that the cholesteryl oleate collapsed phase exists as an isotropic liquid or mesophase below its normal transition temperatures of 51°C (crystalline to isotropic), 47.5°C (isotropic to cholesteric), and 41°C (cholesteric to smectic) (2). It has been shown that cholesteryl oleate can be dispersed into an excess of water as small droplets of approximately 1 μ diameter. These droplets do not show the cholesteric to smectic transition and the smectic

[1] H. L. Brockman, unpublished experiments.

mesophase is stable at temperatures "far below that of the smectic to crystalline transition" (22). This suggests that, in our system at pressures below the triple point (see Fig. 1d–f), the collapsed cholesteryl oleate exists as a smectic mesophase. The author further notes that the suspension particles are virtually identical to spherulites, a lipid mesophase of predominantly cholesteryl esters, which can be isolated from early atherosclerotic lesions (23), and other authors have shown that liquid or liquid crystalline phases are present in more advanced lesions (24, 25). Thus, mixed monolayer systems may be valuable as models for studying the exchange of molecules between the surface and interior of atherosclerotic lesions as well as for studying the interactions of cholesteryl esters with hydrolytic enzymes.

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